Proteome and Secretome Characterization of Glioblastoma-Derived Neural Stem Cells

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Satoshi Okawa[1], Sladjana Gagrica[2], Carla Blin[2,3], Christine Ender[2], Steven M. Pollard[2, 3]*, and Jeroen Krijgsveld[1,4,5]*

[1] European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, Heidelberg 69117, Germany

[2] Department of Cancer Biology, Samantha Dickson Brain Cancer Unit, UCL Cancer Institute, University College London, Paul O'Gorman Building, 72 Huntley Street, London, WC1E 6BT, UK.

[3] Present address: MRC Centre for Regenerative Medicine and Edinburgh CRUK Cancer Research Centre, University of Edinburgh, 5 Little France Drive, Edinburgh, UK, EH16 4UU.

[4] German Cancer Research Center, Im Neuenheimer Feld 581, 69120 Heidelberg, Germany.

[5] CellNetworks - Cluster of Excellence, and Heidelberg University, Heidelberg, Germany

* Correspondence to S.M.P. (email: steven.pollard@ed.ac.uk) or J.K. (email j.krijgsveld@dkfz.de)

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Author contributions:

SO, SMP and JK designed research, SO, SG, CB and CE performed experiments, SO analyzed the data, SO, SMP and JK wrote the manuscript.

Abbreviations

AHA	azidohomoalanine
CSC	cancer stem cell
DAPI	4',6-diamino-2-phenylindole
DTT	dithiothreitol
ECM	extracellular matrix
GBM	glioblastoma multiforme
GNS	glioblastoma-derived neural stem cells
GO	gene ontology
GSEA	gene seat enrichment analysis
IAA	iodoacetamide
IEF	isoelectric focusing
NPC	neural progenitor cells
NS	neural stem cell
pSILAC	pulsed SILAC
SILAC	stable isotope labeling with amino acids in cell culture
TF	transcription factor

Summary

Glioblastoma multiforme (GBM) (grade IV astrocytoma) is the most common and aggressive primary brain tumour. GBM consists of heterogeneous cell types includinga subset of stem cell-like cells thought to sustain tumour growth. These tumour-initiating GBM-derived neural stem (GNS) cells as well as their genetically normal neural stem (NS) counterparts can be propagated in culture as relatively pure populations. Here we perform quantitative proteomics to globally characterize and compare total proteome plus the secreted proteome (secretome) between GNS cells and NS cells. Proteins and pathways that distinguish malignant cancer (GNS) stem cells from their genetically normal counterparts (NS cells) might have value as new biomarkers or therapeutic targets. Our analysis identified and quantified ~7500 proteins in the proteome and ~2000 in the secretome, 447 and 138 of which were differentially expressed, respectively. Notable tumour-associated processes identified using gene set enrichment analysis included: ECM interactions, focal adhesion, cell motility and cell signalling. We focused on differentially expressed surface proteins, and identified 26 that participate in ligand-receptor pairs that play a prominent role in tumourigenesis. Immunocytochemistry and immunoblotting confirmed that CD9, a recently identified marker of adult subventricular zone neural stem cells, was consistently enriched across a larger set of primary GNS cell lines. CD9 may therefore have value as a GNS-specific surface marker and a candidate therapeutic target. Altogether, these findings support the notion that increased cell-matrix and cell-cell adhesion molecules play a crucial role in promoting the tumour initiating and infiltrative properties of GNS cells.

Significance

Glioblastoma multiforme is the most aggressive primary brain tumour, sustained by tumour-initiating glioblastoma-derived neural stem cells. We performed proteomics to identify the molecular signatures that distinguish malignant cancer stem cells from normal neural stem cells, i.e. their non-neoplastic counterparts. By quantitative profiling of the proteins that are expressed and secreted by both cell types across a wide range of patient-derived primary cell lines, we revealed elevated expression of many cell-matrix and cell-cell adhesion proteins in glioblastoma stem cells. In addition, we demonstrated consistently higher expression of the cell surface protein CD9 in glioblastoma stem cells indicating this may have a functional role in conferring GNS specific infiltrative properties, and could also prove useful as a surface marker.

Introduction

Among primary adult brain tumours, glioblastoma multiforme (GBM), also termed grade IV astrocytoma, is the most common and severe form, with a median survival time of only 15 months [1]. GBMs can be initiated in xenograft models following transplantation into immuno-compromised mice of a subpopulation of neural stem cell-like cells derived from patient samples. Such cells are critical therapeutic targets as they likely fuel tumour growth and relapse after therapy [2-4] . GBM stem cell-like cells exhibit functional properties that are shared with normal neural stem cells, such as self-renewal and ability to differentiate. For this reason, tumour-derived stem cell-like cells are commonly referred to as CSCs, or tumour-initiating cells.

Efforts have been made to catalogue genetic aberrations in GBM and associated disrupted signalling pathways [5-7]. However, individual tumours are comprised of varying proportions of immature CSCs and their more differentiated progeny, as well as genetically normal cells (e.g. microglia, tumour-associated macrophages and lymphocytes). Elucidating genes and pathways that drive tumour cell malignancy using genetic and biochemical approaches in bulk tumour cell populations can be misleading, as only an average of molecular signatures can be obtained. It is therefore important to explore glioma pathways specifically within the GBM stem cell compartment.

Ideally, one would compare the genetically normal tissue stem cell with its malignant counterpart under identical experimental conditions. Tumour-specific pathways might then be identified that underlie vulnerabilities that could be targeted therapeutically [8] . ‘Classic’ glioma cell lines fail to provide a realistic disease model, largely due to accumulation of in vitro genetic changes to adapt to the serum culture environment [9] . By contrast, serum-free NS cell culture conditions can be used successfully to enrich and expand brain tumour stem cells either in suspension as ‘spheres’ [2, 4, 10-12] or using adherent monolayer NS cell-culture conditions; the latter providing a more uniform culture environment that suppresses spontaneous differentiation [3]. Karyotypically normal, untransformed foetal NS cells share many features with GNS cells, such as expression of key neural stem/progenitor markers and core transcription factors, such as NESTIN, OLIG2 and SOX2 [3].

Here we search for molecular signatures differentially utilized by the malignant GNS cells compared to genetically normal NS cells. Quantitative proteomics was performed using our recently developed technique for secretome analysis [13] . This significantly extends and refines a previous study that used neurosphere cultures and 2D-gel-based proteomic technologies [14] , and provides a valuable proteomics resource for future glioma studies. We identified GNS-specific signatures, such as differentially expressed secreted proteins, plasma membrane receptors, transcription factors (TFs) and deregulated signalling pathways, which representcandidate biomarkers and therapeutic targets. CD9 emerges from our study as a useful cell surface marker consistently highly expressed in GNS cells compared to NS cells. CD9 has previously been defined as a regulator of cancer cell motility, and recently shown to be a marker of quiescent

astrocyte stem cells in mice [15].

Materials and methods

Cell lines

For the total cell proteome analysis, NS cell lines (CB660, CB192, CB152 and CTX985) were derived from human foetal forebrain (between weeks 7 and 9). GNS lines included (G144, G166, G179 and G25 and cover a diversity of primary GBM subtypes with transcriptional signatures across proneural and mesenchymal phenotypes [16]. Tissues were obtained following informed consent and approval of ethical review panels (reference number 08/H0712/34+5). Full details of NS and GNS cell line derivation and propagation is described elsewhere [3, 17]. For the secretome experiment, CB660, U5 and CB11130 NS cell lines, and G179, G144 and G7 GNS cell lines were used; U5 was used instead of CTX985 as it grew better as a monolayer. The cell lines CB660, CB152, G166, G7 are female, whereas CB11130, G144, G179 are male. Gender of U3 and U5 is not known.

Cell culture

Both NS cells and GNS cells were cultured serum-freein Dulbecco's modified Eagle's medium supplemented with bovine serum albumin (Invitrogen), penicillin/streptomycin (PAA), N2 supplement (PAA), B27 supplement (PAA), EGF (Peprotech), FGF (Peprotech) and laminin (Sigma), as described previously [3, 18] . Culture medium was replaced every 3–5 days. Cells were grown to 60-70 % confluence (2 – 4 million cells), harvested and split 1:3 to 1:4. The passage numbers used for our analysis were p20 for CB660, p27 for CB192, p15 for CB152, p10 for CTX985, p28 for G144, p18 for G166, p18 for G179 and p26 for G25. Our previous studies have noted a risk of in vitro chromosomal aberrations after passage 20 [3]; however, these are mainly whole chromosome gains and losses (i.e. chromosome instability), which is a feature of the disease. Here, we reduce the risk of genetic drift by using multiple cell lines across patient subtypes. Secretome analysis was performed combining enrichment of secreted proteins via click chemistry and SILAC labelling [13, 19]. For stable isotope-labelled amino acid labelling (SILAC) and AHA-labelling for the secretome analysis, cells were grown to 60-70% confluence. Then, the cells were grown either in intermediate media containing Lys-4, Arg-6 (each 1ug/ml) and AHA (0.2 ul/ml), or in heavy media containing 1ul/ml Lys-6, Arg-10 (each 1ug/ml) and AHA (0.2 ul/ml). Supernatant was collected from 2 – 4 million cells cultured in 10 ml of the same medium for 24 h, cleared by centrifugation at 4000 RPM for 10 min before analysis by LC-MS/MS. The viability of the cells after AHA incorporation was assessed with ViCellar. The passage numbers are p10 for CB660, p10 for U5, p14 for CB11130, p40 for G144, p31 for G166 and p30 for G7.

Peptide sample preparation

The cell pellets were homogenized in RapiGest (Waters) followed by reduction of disulphide bonds using 100 mM dithiothreitol (DTT), alkylation using 100 mM iodoacetic acid (IAA) and protein digestion using sequencing grade modified trypsin (Promega) overnight at 37°C. Peptides were stable isotope-labelled via reductive dimethylation, as described in [20]. Peptides were fractionated into 12 fractions on Agilent 3100 OFFGEL Fractionator (settings as described by the manufacturer).Peptides were acidified, desalted with C18 StageTips (Empore 3M), and reconstituted with 4% acetonitrile in 0.1% formic acid. For secretome analysis, 8 ml of each cell supernatant was filtered using Amicon Ultra Centrifugal Filters (3-kDa cutoff) to anend volume of ~250 µL. The proteins in this concentrated media were enriched using the Click-iT Protein Enrichment Kit (Invitrogen C10416) and digested with trypsin as described in [19].

Liquid chromatography-tandem MS (LC-MS/MS)

Peptides were analysed by LC coupled to an LTQ Orbitrap Velos (Thermo Fisher Scientific). Reverse phase chromatography was performed with a nanoACQUITY UltraPerformance LC system (Waters) fitted with a trapping column (nanoAcquity Symmetry C18, 5 µm, 180 µm x 20 mm) and an analytical column (nanoAcquity BEH C18, 1.7 µm, 75 µm x 200 mm). The mobile phases for LC separation were 0.1% (v/v) formic acid in LC-MS grade water (solvent A) and 0.1% (v/v) formic acid in can (solvent B). Peptides were separated at a flow rate of 300 nl/min with a 3 to 40% solvent B gradient for 145 min for each IEF fraction. The MS1 scan was acquired in the Orbitrap from m/z 300 to 1,700. Fragmentation was performed in the LTQ by CID, selecting up to 15 most intense ions (top15) at an isolation window of 2 Da.

Data processing

Raw files were processed with MaxQuant [21] version 1.2.0.17 and the Andromeda search engine [22] . The MS/MS spectra were searched against the Human UniProt database (downloaded on June 21, 2011) containing 69906 forward sequences which was appended to the same number of reverse sequences and 265 common contaminants. The precursor mass tolerance was set to 20 ppm for the first pass and 6 ppm for the 2^{nd} pass. The fragment mass tolerance was 0.5 Da. Quantification was done by DimethylLys0 + DimethylNter0 for the light labelling and DimethylLys4 + DimethylNter4 for the intermediate labelling. Unique- and razor peptides were used for quantification. Cysteine carbamidomethylation and methionine oxidation were set for the fixed modification and variable modification, respectively. In case of AHA experiments, methionine was replaced with AHA. The minimum peptide length was set to 6 amino acids, the enzyme specificity was set to trypsin/P, the maximum allowed miss-cleavage was set to 2, and the false discovery rate (FDR) was set to 0.01 for both peptide and protein identifications. Re-quantification and match between runs were also performed. The protein identification was reported as a “protein group” if no unique peptide sequence to a single database entry was identified. The data were deposited to

Chorus (https://chorusproject.org/pages/dashboard.html#/projects/all) under the project title 'Proteome and secretome characterisation of normal and glioblastoma-derived neural stem cells' (project ID 723). After MaxQuant processing, reverse and contaminant proteins and proteins with only one peptide identification were discarded. MaxQuant-computed raw protein ratios were normalized with median and median absolute deviation (MAD) using the following scheme:

$$X_j = \frac{X_j - median}{MAD}$$

where X_j is the raw protein ratio of protein *j*.

Differential expression analysis

To determine differential protein expression we only considered proteins quantified in all the four GNS samples, split the data into intensity bins each containing 300 proteins to account for dependence of ratio variance on the intensity. Significance for differential protein expression was tested for each bin by a moderated t test using the limma R package [23] and correction for multiple testing by the Benjamini-Hochberg method with an FDR threshold of 0.05. Subsequently, protein groups with expression value less than 2-fold were discarded. Cell line-specific differentially expressed protein groups were defined as 1) those that had expression ratios less than 2-fold (up or down) in the cell line in question but more than 4-fold in the other three cell lines, or 2) those that had expression values more than 4-fold (up or down) in the cell line in question but less than 2-fold in the other three cell lines in the reverse expression direction.

Transcriptome data

The Tag-Seq data of NS and GNS cell lines was obtained from our previous study [24] including 485 upregulated and 254 down-regulated genes in GNS cells relative to NS cells. The passage numbers of the cell lines used in this study were between 10 – 20.

In silico protein functional analysis

The annotation of “transcription factor/regulator” was retrieved from Panther, MetaCore (GeneGo Inc.) and UniProt keywords (http://www.uniprot.org/keywords/). Transcription targets were retrieved from MetaCore, and prior association with glioma stem cells, glioma cells and neural stem cells was investigated by PubMed literature search. Gene set enrichment analysis (GSEA) of differentially expressed proteins were subjected to a Fisher's exact test for the chromosomes, Gene Ontology (http://www.geneontology.org/) biological processes (GO.BP), Panther pathway, Reactome Pathway/Complex, WikiPathway, CORUM, and KEGG using all quantified proteins as the background and correcting for multiple testing by the Benjamini-Hochberg method. Computational prediction of secreted proteins was performed using SignalP

(http://www.cbs.dtu.dk/services/SignalP/), and by UniProt including proteins with the keywords “Signal”, “Secreted” or “Extracellular space”. If a protein belongs to at least one of these four criteria, that protein is considered “predicted to be secreted” otherwise “not predicted to be secreted”. Known interactions between ligands and receptors were retrieved from MetaCore. Plasma membrane receptors were defined as those having either the UniProt keyword “cell membrane” or Panther protein class “receptor” or GO.CC “plasma membrane”. Self-interactions were discarded.

Network analysis

Protein-protein and protein-DNA interactions and their 1st neighbours were retrieved using Cytoscape [25] plugin BisoGenet [26] further expanded by CytargetLinker [27] for transcriptional interactions from ENCODE and TFe (http://www.cisreg.ca/cgi-bin/tfe/home.pl). From this network, all the enriched processes from the GSEA as well as differentially expressed proteins were extracted. The resultant subnetwork was then processed with jActiveModule [28] in order to extract a subnetwork of low adjusted p-values using the simulated annealing algorithm.Direct interactions among the differentially expressed proteins and mRNAs were retrieved from MetaCore. The network edges (i.e., interactions) were pruned using the method developed by Crespo et al [29] , visualizing the best network configuration using the Cytoscape plugin BiNoM (version 2.5) [25].

Immunoblotting

Cells were lysed in 150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.5% NP-40, 2 mM EDTA, 1 mM NaF and protease inhibitors. Primary antibodies used were: Tenascin-C (1:100, Sigma), Galectin-3 (1:100, abcam), CD9 (1:100, Millipore), and GAPDH (1:1000, GeneTex).

Immunocytochemistry

Cells were fixed in 4% paraformaldehye (PFA) for 8 min,washed/permeabilised (1× PBS+0.1% Triton-X 100) and blocked for 1 h inwash buffer plus 3% goat serum and 1% BSA. Primary antibodies were incubated overnight at 4 °C,washed for 2 x 5 min followed by incubation with secondary antibodies for 1 h at room temperature. Images were taken using an Olympus IX50 inverted fluorescent microscope with a DP-50 camera. DAPI was used as a nuclear counterstain. Species-specific or Ig-subtype specific goat secondary antibodies were used throughout with either Alexa488 or Alexa594 fluorophores (Molecular Probes/Invitrogen). Fluorescence images were quantified in ImageJ. The CD9 fluorescence signal was thresholded in order to create a binary mask delineating the contours of individual cells. When cells were too close from one another to be properly separated, a boundary between the cells was drawn manually. Disconnected areas of the binary mask were then labelled with a unique identifier (Analyse Particle plugin) and the mean gray level intensity of the CD9 signal was measured for each labeled area.

Results

Differential protein expression and secretion between NS and GNS cells

To reveal the differences in protein expression between NS cells and GNS cells, we performed an in-depth proteomics analysis. The experiment was set up such that four individual GNS cell lines (G144, G166, G25, G179) [30], covering a diversity of primary GBM subtypes (not IDH1/2 mutant), were each compared to a common reference consisting of a pool of four NS cell lines (CB660, CB192, CB152 and CTX985) (Figure 1A), thus allowing us to identify proteins that are consistently differentially expressed between NS and GNS cells, as well as those that differ between individual GNS lines. This required 120 LC-MS/MS runs, quantifying 7476 proteins, of which 6492 were quantified in all the samples (Suppl Table T1). The mean protein ratio correlation between replicates applying reverse isotope labelling was 0.62 (Figure S1). The mean sample correlation coefficient based on protein ratios quantified in all the samples was 0.38 (Figure S2), suggesting extensive differences between the GNS cell lines. There were 136, 110, 59 and 79, cell line specific differentially expressed proteins in G166, G144, G25 and G179 lines, respectively. The differential abundance test between the four GNS cells and the pooled NS sample yielded 465 differentially expressed proteins (Figure 1B and Suppl Table T1), 447 of which were present in all four GNS cell samples (155 were higher in GNS cells; 292 were higher in NS cells). We focused on these 447 differentially expressed proteins in the subsequent analyses (Suppl Data set D1). In parallel we assessed the secreted proteome for each of three GNS lines (G144, G166 and G7) compared to a common reference consisting of the pooled secretome of 3 NS cell lines (CB660, U5, and CB11130) (Figure 1A). The mean protein ratio correlation between biological replicates was 0.86 (Figure S3) with a mean sample correlation coefficient based on protein ratios quantified in all the samples of 0.6 (Figure S4). 1718 proteins were quantified across all the three samples, of which 363 were differentially expressed (Suppl Table T1). Among the 389 proteins with predicted secretion (see Materials and methods), 138 were differentially expressed (122 up- and 16 down-regulated). 225 differentially expressed proteins were not predicted to be secreted (135 up and 90 down) (Suppl Data set D2).

The volcano plot of differentially secreted proteins shows that GNS cells tend to secrete more proteins than NS cells (Figure 1C). The small overlap of differentially expressed proteins from the characterised total proteome and secretome (37 proteins) suggests that our secretome experiment was able to capture proteins which were not differentially expressed in the total cell experiment, most likely due to differential secretory activity for specific proteins (Figure S5). The full details of all quantified proteins from the proteome and secretome experiments are listed in Suppl Data sets D1 and D2, respectively.

Gene set enrichment analysis (GSEA) captured known chromosomal aberrations and putative tumour-associated processes

The 447 differentially expressed proteins consistently differentially expressed across the four GNS cell lines were subjected to GSEA to gain insight in over-represented biological processes (Table 1 and Suppl Data set D3). At the chromosome level, proteins expressed from chromosomes 7 and 15 were over-represented, while those from chromosome X were under-represented. This is consistent with the known gains of chromosome 7 and loss of 15 in GBMs and GNS cell lines [3, 24]. Indeed, when plotting protein ratios between NS and GNS cells along all chromosomes, protein expression of chromosome 7 was increased, whereas that of chromosome 15 was decreased in each of the cell lines (Figure 2A). In addition, Figure 2A shows differential protein expression from distinct chromosomal regions in individual cell lines, e.g. increased expression from chromosome 19 in G166, and decreased expression from chromosome 10 in G25 suggesting additional chromosome aberrations. The under-representation of X could be due to the fact that GNS lines contained fewer females than in NS lines. The clear detection of known glioma associated karyotype abnormalities from our data gave us confidence in the approach.

The over-representation of Gene Ontology Biological Processes “cell differentiation” and “neuron differentiation” (Table 1) may relate to tumour-specific abrogation of differentiation commitment and mitotic arrest. Enrichment of cell motility-related categories such as regulation of cell migration, cell junction organization and gap junction could be explained by the increased cell motility and invasive property of GNS cells. In addition, several integrin-related categories were enriched, possibly reflecting their known role in the ECM to modulate cellular shape, motility and cell cycle progression [31] . Integrin-ECM interactions are also well known to be important components of stem cell niches across diverse tissues. Furthermore, interactions between the ECM and the actin cytoskeleton commonly take place at focal adhesions. In agreement with this, ECM-, focal adhesion- and cytoskeleton related processes were over-represented (Table 1), including proteins such as collagens, MAPKs, integrins, SRC and RRAS, suggesting the importance of ECM re-organization for tumourigenesis, a property that is apparently maintained even in cultured cells.Detection of several notable signalling pathways with previously known roles in glioma including ERK1 activation, negative regulation of TGF-beta receptor signalling pathway, positive regulation of calcium-mediated signaling, indicates that these pathways remain active in vitro and that their disregulation could be associated with tumourigenicity. Endocytosis is known to digest ECM components such as Cadherins and its over-representation in our GNS cells may suggest an enhanced microvesicle transport of RNA and proteins, which was previously reported in glioma cells [32] . The GSEA using CORUM and ReactomeComplex resulted in largely a similar result with many integrin- and focal adhesion-related complexes (Suppl Data set D3). Taken together, our analyses captured known chromosomal aberrations and many biological pathways and processes that could be responsible for GNS malignancy, and identified altered integrin-mediated signalling as the most notable difference between normal NS and GNS cells.

Expression of known glioma stem cell markers between GNS and NS cells

In our data set we examined expression of proteins that have been defined as glioma stem cell-associated markers in recent years (Figure 2B and [33, 34]). We found that LGALS3, LGALS3BP, L1CAM and GFAP were consistently over-expressed, while ALDH2 and ITGA6 were under-expressed in GNS cells (Figure 2B). LGALS3 is an adult astrocyte stem cell marker known to have many functions [35] ; L1CAM was shown to partially regulate the DNA damage checkpoint response and radio-resistance of glioma stem cells through the activation of the ATM kinase pathway [36].

PROM1 (CD133) has been shown to be a marker of glioma cells both *in vitro* and *in vivo* [37] . Although PROM1 has been used to isolate brain tumour stem cells [4, 38] , it was not differentially expressed when compared to the NS cells (also not on the mRNA level [24]), suggesting that this marker may be shared between NS and GNS cells. GFAP is an astrocyte marker, but is also expressed by quiescent adult neural stem cells and foetal radial glia in humans [3]. Finally, ITGA6 is a receptor for the ECM protein laminin and its mRNA was highly expressed in embryonic, neural, and heamatopoietic stem cells [39]. In summary, several known glioma stem cell markers were differentially expressed between NS cells and GNS cells.

Differential expression of TFs in NS and GNS cells

In our data we identified 36 TFs that were consistently differentially expressed in all four GNS cell lines compared to NS cells, 19 of which were up-regulated and 17 were down-regulated (Suppl Table T2). They include anticipated tumour suppressors (e.g. TP53, YAP1) and cell cycle regulators (LZTS1, HMGA2). Eight of these have been implicated in glioma (Suppl Table T2), while others have been associated with other cancers. HMGA2 was under-expressed in GNS cells, consistent with data at the mRNA level [24] and with its low expression or absence of mRNA in glioblastoma tissue in comparison to low-grade gliomas [40] . LZTS1 is known to activate CDK1 by physical interaction, however it has not previously been linked to glioma (Suppl Table T2). Finally, STAT3 was not among the 447 differentially expressed proteins, yet it was consistently under-expressed in all the four GNS cell lines (mean log2 ratio -0.98, adjusted p-value 0.03). STAT3 was implicated in maintaining the stemness of glioma stem cells [41] , however its down-regulation in our GNS compared to NS cells might indicate its role specifically in tumourigenesis, rather than stem cell-related properties.

Comparison of proteomics data with transcriptome data in GNS and NS cells

Since a comparison of differentially expressed mRNAs between GNS and NS cells was previously reported [24], we intersected this transcriptome data with our new proteomic data. We identified 34 gene products showing consistent up- or down-regulation (15 up, 19 down regulated) both at the mRNA and protein levels (Figure 2C). The set of up-regulated proteins/mRNAs included the

above-mentioned GFAP and LGALS3, and NNMT and CD9. Notably, NNMT was previously reported as up-regulated in glioma stem cells in comparison to normal NS cells [14] . CD9 is a tetraspanin found in exosomes and has been shown to modulate cell migration and tumour metastasis [42] . Among the gene products down-regulated at both protein and transcript levels, TAGLN (Transgelin) has been characterised as a tumour suppressor gene in non-brain tissues [43] . In contrast, many genes were differentially expressed only at the protein level and therefore could not have been detected with transcriptome profling studies. Of note, FOXO3 was up-regulated in GNS cells even though mRNA showed no differential expression (mean log2 ratio 0.02, adjusted p-value 1), and it may therefore undergo post-transcriptional regulation.

Classification of differentially secreted proteins

We next defined two sets of differentially expressed proteins from the secretome experiment; Set 1 contains proteins predicted to be secreted and Set 2 contains those not predicted to be secreted (see Materials and method for details). Set 2 can be important since many secreted proteins have been shown to lack a signal peptide for the classical endoplasmic reticulum (ER)-Golgi pathway, and are secreted via unconventional pathways [44] . Indeed, among the 229 proteins in Set 2, 170 were either predicted to be unconventionally secreted by SecretomeP 2.0 [45] or found in exosomes [46]. Proteins in Set 1 and Set 2 were functionally categorized using UniProt keywords and keywords that are likely to be related to tumourigenesis (Suppl Figure S6). The top three most abundant keywords in Set 1 were "Receptor / membrane protein", "Cell adhesion" and "EGF-like domain", whereas those in Set 2 were "Receptor / membrane protein", "Protease / protease inhibitor" and "Differentiation". The large number of receptor / membrane proteins suggests that many of these proteins are shed to the extracellular space. Additionally, we found total 31 differentially expressed proteases / protease inhibitors, including serine proteases (SERPINB6 and SERPINB8), metalloproteinase-related proteins (ADAM10, TIMP1, TIMP2 and TIMP4) and cathepsins (CTSA and CTSD), possibly involved in matrix degradation and tumour cell invasion and migration, a well-known feature of highly infiltrative GBM cells [47-51].

Growth factors and cytokines constitute another important class of signaling proteins that are difficult to capture in total cell proteome. Our secretome data contained 10 differentially expressed proteins in this category (CAT, CSF1, GRN, IGFBP4, LTBP4, NOV, PRNP and VEGFA (over-expression), IGFBP3 and IGFBP5 (under-expression)), primarily known to promote cell proliferation. 26 proteins belonged to the category “Cell adhesion” (Suppl Figure S6) and as such may be related to cancer-cell invasion. This group includes proteins with (e.g. L1CAM, MCAN, NID2) and without prior association with glioma (e.g. FREM2, LGALS3BP, FLRT3). Taken together, our secretome analysis provides a valuable complement to the data on the cellular proteome, identifying many extracellular proteins with an enzymatic or signaling function. Several of these are known to play a role in glioma, but this also includes several novel candidates that may be investigated in future functional studies.

Interaction between differentially expressed secreted proteins and receptors identified potential GNS markers

Next we explored known interactions between differentially expressed ligands (in the secretome) and differentially expressed plasma membrane receptors (in the total proteome), reasoning that this combined data may identify specific ligand-receptor pairs associated with GNS cell properties. Of the147 interactions (Suppl Data set D4), we particularly focused on pairs where both ligand and receptor expression changed in the same direction, as they are likely to be signatures of downstream expression changes. We identified 37 such pairs, 26 and 11 of which were up-regulated and down-regulated in GNS cells, respectively (Table 2). Among these, some play a prominent role in tumourigenesis, such as "cell adhesion" (ADAM10-CD9, LGALS3BP-CD9, ADAM10-L1CAM, L1CAM-NCAM1) and "immune system process" (PRNP-C1QBP, ADAM10-L1CAM, CSF1-MYD88, PRNP-L1CAM, CANX-L1CAM, TXN-MYD88, CANX-NDRG1 and B2M-TFRC). Several of these proteins have been highlighted above, and they may now be positioned more firmly in the context of distinct pathways that are specific for GNS cells.

Molecular interaction networks show proteins central to the difference between NS cells and GNS cells

In order to find which proteins are collectively representing the differences between GNS cells and NS cells in the context of molecular networks, we used Cytoscape plugins, BisoGenet and CytargetLinker to retrieve known protein-protein and protein-DNA interactions based on differentially expressed proteins from both total proteome and secretome experiments and all proteins in the enriched processes from the GSEA (Table 1). Then, the sub-network of low adjusted p-values was inferred using jActiveModule, resulting in a network of 276 proteins with 539 interactions (Figure 3A). This unbiased approach recapitulated the differentially expressed proteins, and proteins from the enriched processes (integrins, signalling, adhesion molecules, cytoskeltal proteins). On top of those, the network contained 12 proteins with >50 network degrees (Suppl Table T3), suggesting that these are important in mediating interactions critical to the differences between NS cells and GNS cells. Indeed, all of them have been previously implicated in glioma and cellular proliferation (e.g. BRCA1, BCL3, SP1, GSK3B, PBX3, SMARCB1). Since expression of many of these 12 proteins did not change significantly, this provides a complementary approach to identifying biologically important proteins.

Additionally, to infer a gene regulatory network that could underlie the difference between GNS and NS cells, differentially expressed proteins and mRNAs from [24] were also analysed using the previously proposed method [29]. This identified a gene regulatory network core (Figure 3B) that could best explain the differential expression between GNS cells and NS cells. Reassuringly, this network included several well-known tumour-associated proteins such as PDGFRA, EGFR, TP53, and MYC, which are the major genetic drivers of tumourigenesis in

glioblastoma, as well as CD9 and TNC (tenascin C).TNC is an ECM glycoprotein that is abundantly expressed in foetal NS cells and vanishes as the organism matures and is absent in normal adult brains [52] but re-expressed upon injury or neoplasia [53, 54]. In addition, cytotoxic-antibodies against TNC have been under clinical trials [55]. This agrees with our data where TNC shows elevated expression in GNS cells compared to NS cells.

Immunocytochemistry and immunoblotting confirm CD9 enrichment in GNS cells compared to NS cells

Our bioinformatics analyses aimed to catalogue the differences between GNS cells and NS cells in an unbiased manner. We next shortlisted genes that appeared at least twice in different analyses. We particularly focused on cell-surface proteins since they are potential mediators of signalling or protein secretion, and that may be accessible for cell sorting and future therapeutic targets. From these considerations, four candidates emerged (TNC, THY1, LGALS3 and CD9 (Figure 4A). CD9 is of particular interest given its recent identification as a potential endogenous NS cell marker in mice [15]. Western immunoblotting was performed for TNC, LGALS3, and CD9 using a larger set of lines (Figure 4B). We found no clear differential expression for TNC with the antibodies used, and LGALS3 failed to be confirmed as consistently enriched in GNS cells across a larger panel of lines. However, by both immunocytochemistry and western immunoblotting we were able to confirm that CD9 is consistently over-expressed in GNS cells, compared to NS cells (Figure 4C, D) demonstrating that CD9 can distinguish these cell types. Finally, apatient survival analysis was performed over large cohorts of primary GBM tumours usingthe Rembrandt database (http://www.betastasis.com/glioma/rembrandt/). This revealed a significant negative correlation between the CD9 expression levels and overall patient survival (Figure 4E),suggesting that it may have value as a GNS-specific marker.

Discussion

It has been recognized that only a subset of cells within GBM is able to reconstitute a tumour and is therefore responsible for driving tumour development in vivo [2-4]. These stem cell-like cells (i.e. CSCs) share similar functional properties with normal stem cells including the abilities to self-renew and differentiate. As cells regulate themselves and nearby cells through the niche by secreting auto/paracrine cues, not only intracellular proteins but also proteins secreted from these cells are likely to contribute to the difference between malignant and normal stem cells. To reveal such differences in protein expression between untransformed, karyotypically normal foetal NS cells and malignant NS cells derived from adult gliomas (GNS cells), we have performed mass spectrometric analyses of both total cell proteomes and secreted proteomes. We used cells expanded in adherent monolayer culture, which provides a more uniform culture environment with reduced

differentiation and apoptosis that accompany suspension cultures [3]. The direct comparison between NS and GNS cells removes those set of genes that are shared stem cell-related properties present in both cell types, enabling us to focus on protein repertoires distinguishing non-malignant NS cells from highly malignant GNS cells. Comparisons between normal stem cells and CSCs is an important research question, as this knowledge might be useful for specifically targeting CSCs, while minimizing the effect on the normal counterparts [8].

The result showed that, as anticipate there are striking differences between each patient GNS cell line, mirroring the distinct variants of the human disease. However, by focusing on proteins differentially expressed across all GNS cell lines tested, we aimed to derive 'core' malignant properties shared across different individuals and across the genetic diversity.At the same time, this would equalize differences between cell lines arising from potential spatial or temporal variation of the collected tissues or cell of origin. The generated proteomics and secretome data and our previous transcriptome data comparing GNS cells with NS cells [24] providing a compendium of differentially expressed proteins; this was used to perform a multi-layered bioinformatic analysis to define most significantly utilized tumour-enriched pathways. Several patterns emerged from this analysis that partly confirmed existing literature, while extending the panel of GNS-specific proteins with novel candidate markers and functional regulators (Figure 5). For instance, among the known NS cell and glioma stem cell markers, LGALS3, LGALS3BP, L1CAM, GFAP were more highly expressed in GNS cells, while expression of ITGA6 and ALDH2 was reduced. These markers are expressed by quiescent niche-dependent adult stem cells, and maybe highlight the ability of glioma genetic drivers to impose aberrant proliferation of both the quiescent cells (type B-like cells) as well as the activated rapidly dividing stem and progenitors (type C cells) [56]. Additionally, 36 transcription factors/regulators were differentially expressed, 9 of which (FOXO3, TP53, IFI16, NFIC, PURA, YAP1, HMGA2 and PRKCB) have been previously implicated in glioma, whereas the other 27 are unreported.

The GSEA of the 447 differentially expressed proteins showed several tumour-associated processes, including those related to ECM interactions, focal adhesion, cell motility, structural organization and cell signalling. These findings suggest that aggressive tumour initiation and infiltration of GNS cells is explained by these sets of proteins being expressed. The clear difference in regulation of ECM could also support the notion that a tumour-specific niche is created by tumours with increases in adult quiescent stem cell markers such as LGALS3 and GFAP. This would enable for self-renewal to be sustainedby the GNS cells outside of the endogenous SVZ niche – likely protecting the GBM stem cell from pro-differentiation cues.

In our secretome analysis a striking observation was that the majority of differentially expressed proteins were preferentially secreted by GNS cells (122 out of 138). This trend was also observed in the comparison between cancer and normal cells in our previous work [13]. Many of these differentially secreted proteins belong to the tumour-related functionalities such as cell adhesion, protease / protease inhibitor and growth factor / cytokine / mitogen. The fact that

proteases and growth factors were abundant in the differentially expressed secretome proteins but not in the differentially expressed proteins in the total cell experiment indicates the importance of analysing the extracellular environment to identify proteins with a potential role in shaping the tumour micro-environment.

Beyond changes in protein expression levels we also considered protein connectivity in interaction networks to estimate their potential impact on associated cellular processes, reasoning that a change in the expression of proteins that formed the regulatory core have a high influence on the cellular phenotypes. This revealed, for example, several well-known players in regulating processes related to cell proliferation, in particular in cancer cells (BRCA1,BCL3, GSK3B, PBX3, SMARCB1). Furthermore, by combining these bioinformatics approaches with the differentially abundant receptor-ligand pairs and our previous transcriptome data, we identified potential GNS markers that could be distinguished from normal NS counterparts, including CD9. Western blot and immunofluorescence analysis confirmed that CD9 was significantly highly expressed in the GNS cells in comparison to the NS cells. CD9, a transmembrane protein of the tetraspanin family, has been variedly associated with pro- or anti-metastatic properties depending on cellular context and tumor entity, determined in part by interactions with other membrane-embedded signaling proteins (reviewed in [57]). Importantly, recent evidence has indicated that CD9 itself supports maintenance of glioblastoma stem cells [58]. Our data demonstrating that CD9 is consistently higher expressed in GNS cells compared to NS cells provides further evidence for the functional relevance and potential practical utility of CD9, such as sorting of GNS cells from NS cells, or as a GNS-specific target of drugs and antibodies. Interestingly, our secretome analysis identified 3 CD9-ligands (ADAM10, LGALS3BP and PTGFRN, Table 2), which were all up-regulated, and each of which has been linked to metastasis [59-61], including in glioma and glioblastoma. This provides one example how quantitative proteome and secretome analysis offers a powerful means to investigate protein expression as well as the proteins involved in extracellular signaling and that crucially determine downstream effects.

Altogether our proteomics data enabled us to globally characterize molecular signatures that are differentially active/inactive between GBM stem cells and normal neural stem cells, and will be a valuable resource for future glioma studies.

Data availability: all mass spectrometry data associated with this study can be downloaded from Chorus (https://chorusproject.org/pages/dashboard.html#/projects/all) under the project title 'Proteome and secretome characterisation of normal and glioblastoma-derived neural stem cells' (project ID 723).

Acknowledgement

We greatly thank the EMBL proteomics core facility for technical support, Bernd Fischer for

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Conflict of interest

None.

Legends of Tables and Figures

Table 1. Enriched GSEA categories among differentially expressed proteins between GNS and NS cells.

(**A**) chromosomes, (**B**) Gene Ontology Biological Process (GO.BP), (**C**) Panther Pathway, (**D**) Reactome Pathway, (**E**) WikiPathway and (**F**) KEGG. Annotation: total number of genes in category, differentially expressed genes: number of differentially expressed (DE) genes, padj: Fisher's exact test p-value adjusted with Benjamini Hochberg correction.

Table 2. Interactions between differentially expressed ligands and receptors.

Interactions between ligands and receptors that were consistently up- or down-regulated in the secretome and total proteome, respectively, as retrieved from MetaCore. Each interaction was manually validated from references in the literature.

Figure 1

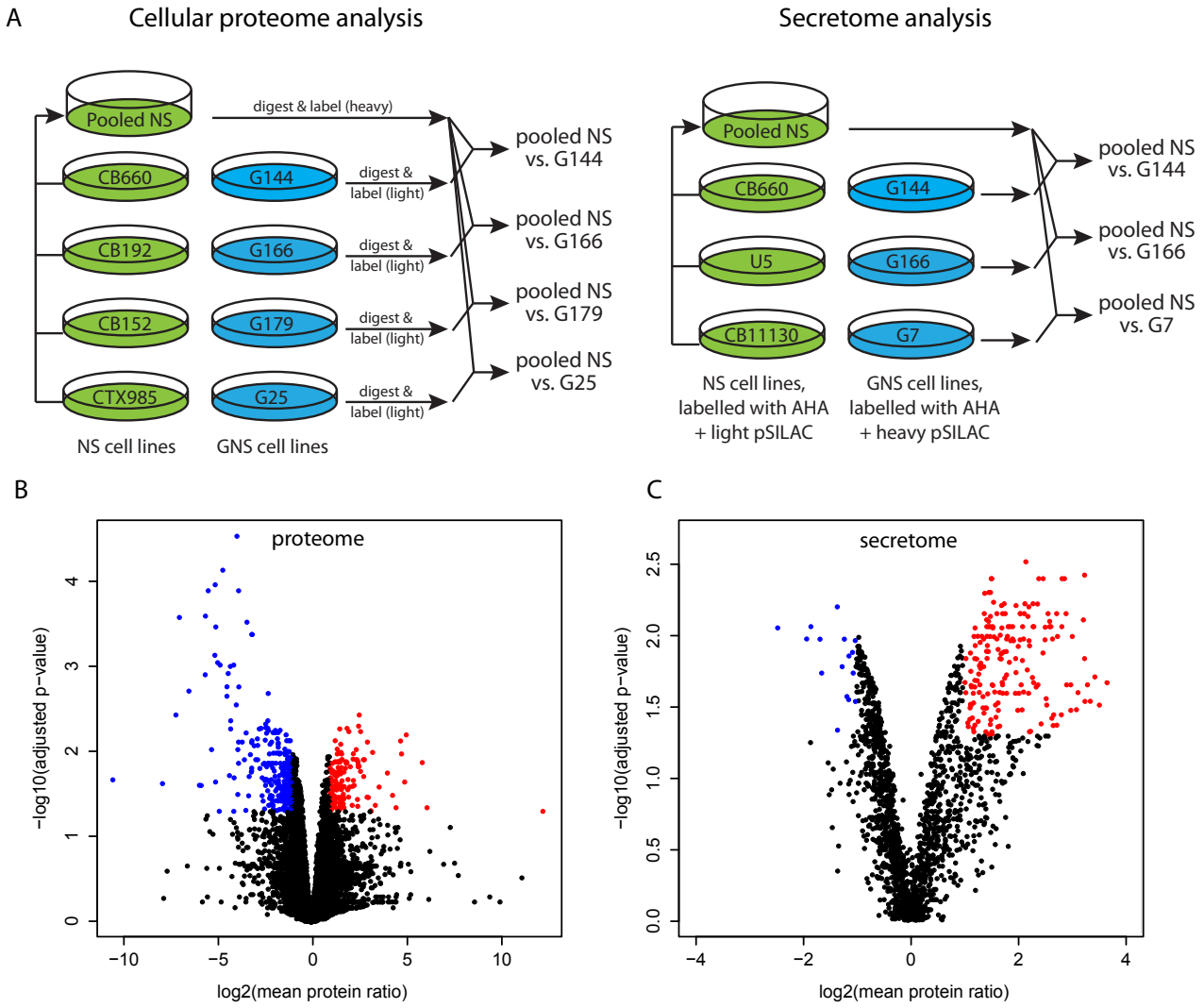
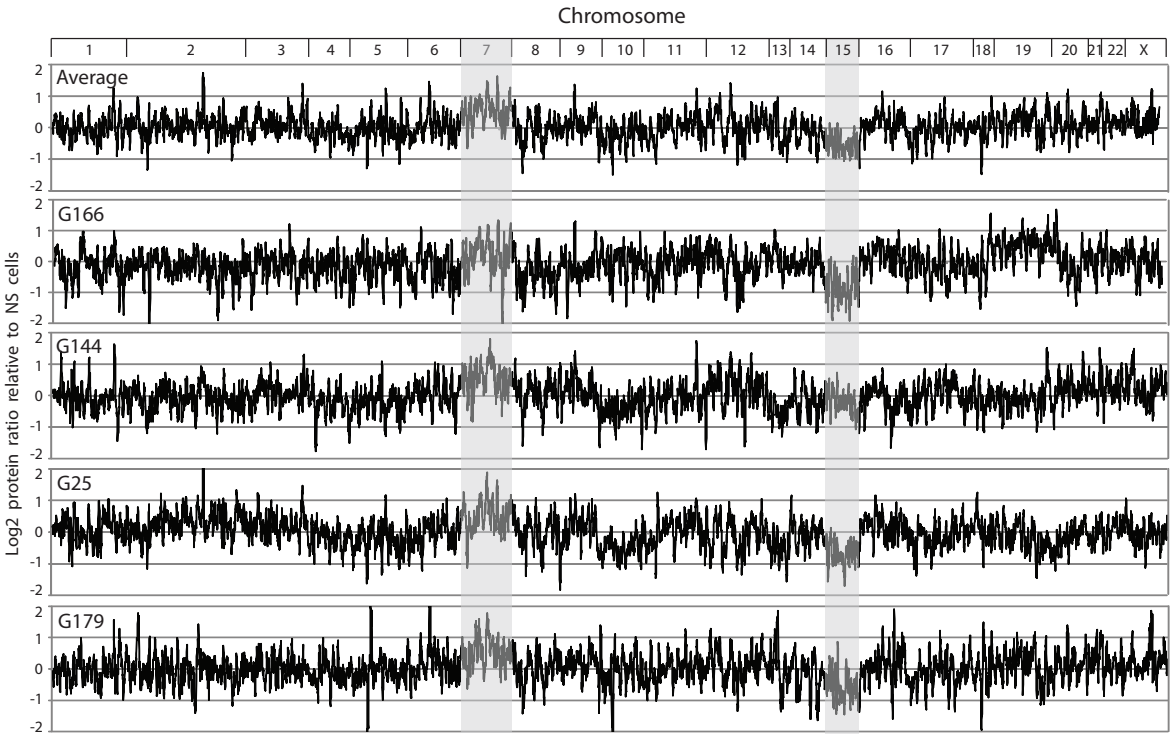
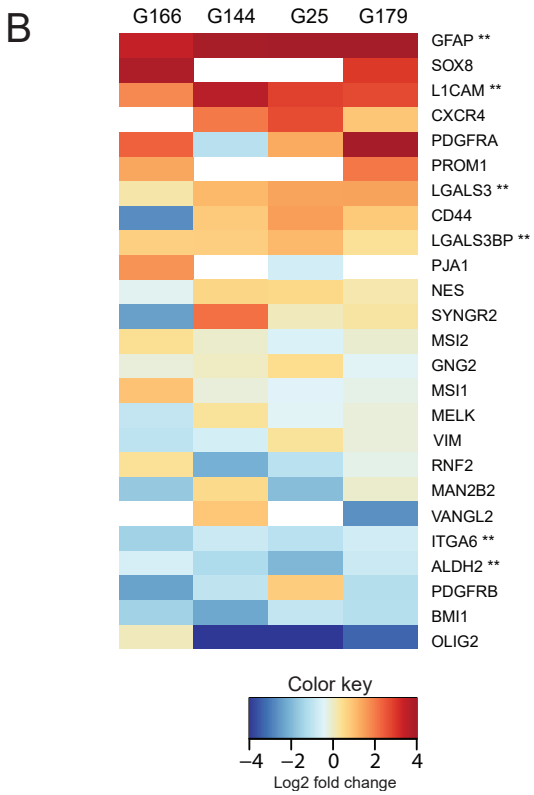

A
Cellular proteome analysis
Secretome analysis
Pooled NS
CB660
CB192
CB152
CTX985
digest & label (heavy)
G144
G166
G179
G25
digest & label (light)
pooled NS vs. G144
pooled NS vs. G166
pooled NS vs. G179
pooled NS vs. G25
NS cell lines
GNS cell lines
U5
CB11130
G7
pooled NS vs. G7
NS cell lines, labelled with AHA + light pSILAC
GNS cell lines, labelled with AHA + heavy pSILAC
B
proteome
−log10(adjusted p−value)
log2(mean protein ratio)
C
secretome

Figure 1. **Quantification of GNS total cell proteome and secreted proteome.**

(**A**) Overall experimental design for the analyses of cellular proteomes and secretomes comparing GNS to NS cells. (**B**) Volcano plot of protein ratios (GNS/NS) for total cell experiment. (**C**) Volcano plot of protein ratios (GNS/NS) for secretome experiment. Red and blue dots in panel B and C indicate proteins that are up- or downregulated, respectively, at least 2-fold and with statistical significance (adj-p value <= 0.05).

Figure 2


A
Chromosome
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Average
G166
G144
G25
G179
Log2 protein ratio relative to NS cells


B
G166
G144
G25
G179
GFAP **
SOX8
L1CAM **
CXCR4
PDGFRA
PROM1
LGALS3 **
CD44
LGALS3BP **
PJA1
NES
SYNGR2
MSI2
GNG2
MSI1
MELK
VIM
RNF2
MAN2B2
VANGL2
ITGA6 **
ALDH2 **
PDGFRB
BMI1
OLIG2
Color key
−4
−2
0
2
4
Log2 fold change

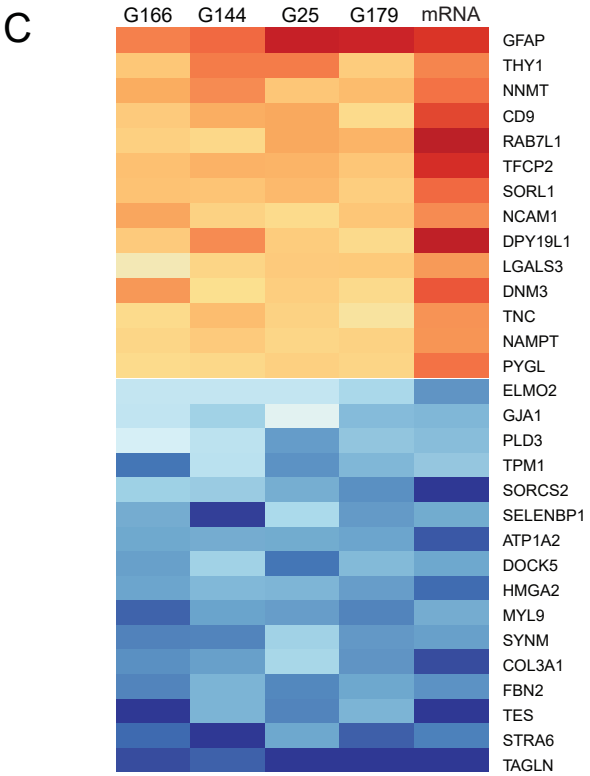
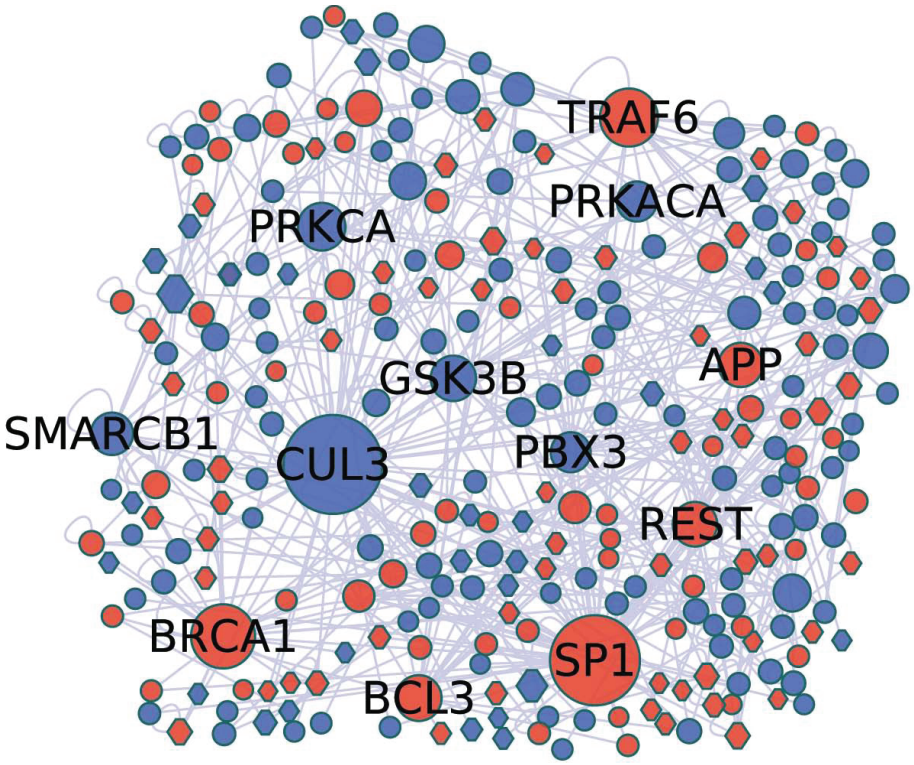

C
G166
G144
G25
G179
mRNA
GFAP
THY1
NNMT
CD9
RAB7L1
TFCP2
SORL1
NCAM1
DPY19L1
LGALS3
DNM3
TNC
NAMPT
PYGL
ELMO2
GJA1
PLD3
TPM1
SORCS2
SELENBP1
ATP1A2
DOCK5
HMGA2
MYL9
SYNM
COL3A1
FBN2
TES
STRA6
TAGLN

Figure 2. Differential protein expression Heatmap of protein expressions from different criteria.

(**A**) Protein ratios as a function of chromosomal locations in each of four GNS cell lines relative to a pool of NS cell lines.The top trace displays the average ratio of all four GNS cell line. Solid black line is a moving average of 10 ratios. Chromosome number is indicated on top. (**B**) Heatmap of known NS and/or GNS marker expression. Asterisk indicates differential expression with statistical significance. Proteins indicated with ** were consistently differentially regulated across all 4 cell lines. (**C**) Heatmap of proteins that were up- and down regulated on both protein and mRNA levels. The colour key is the same as B. The last column (mRNA) is mean log2 fold-change(GNS/NS) of mRNA expressions in cell lines used in [24].

Figure 3

A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PRKCA
APP
GSK3B
SMARCB1
CUL3
PBX3
REST
BRCA1
SP1
BCL3

B

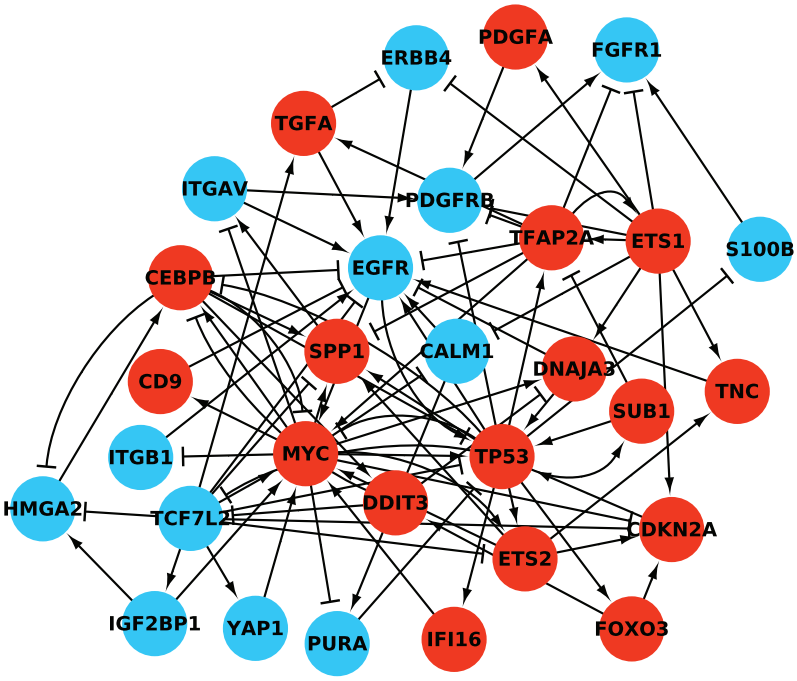

PDGFA
ERBB4
FGFR1
TGFA
ITGAV
PDGFRB
TFAP2A
ETS1
S100B
EGFR
CEBPB
SPP1
CALM1
DNAJA3
CD9
TNC
SUB1
ITGB1
MYC
TP53
HMGA2
DDIT3
TCF7L2
CDKN2A
ETS2
IGF2BP1
YAP1
PURA
IFI16
FOXO3

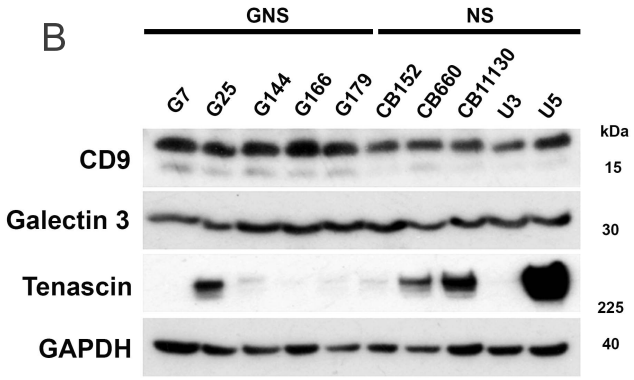
Figure 3. Network analysis of differentially expressed proteins.

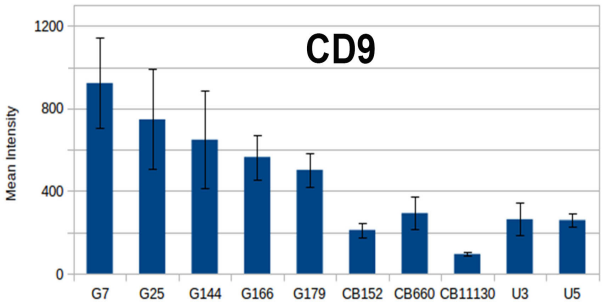
(**A**) A network of 276 proteins created with Cytoscape plugin jActiveModule with adjusted p-value as input parameter. Protein-protein, protein-DNA and transcriptional interactions were retrieved from various databases using Cytoscape plugins BisoGenet and CytargetLinker. Each node represents a protein, while the shape of the node indicates if it originates from total proteome (circles) or secretome data (diamonds). Nodes were colour-coded by negative fold-change (blue) to positive fold-change (red) in GNS cells. Node size indicates the degree (number of connections). For clarity, gene symbols are shown only for those with >50 degrees. (**B**) A gene regulatory network inferred from differentially expressed proteins and mRNAs.

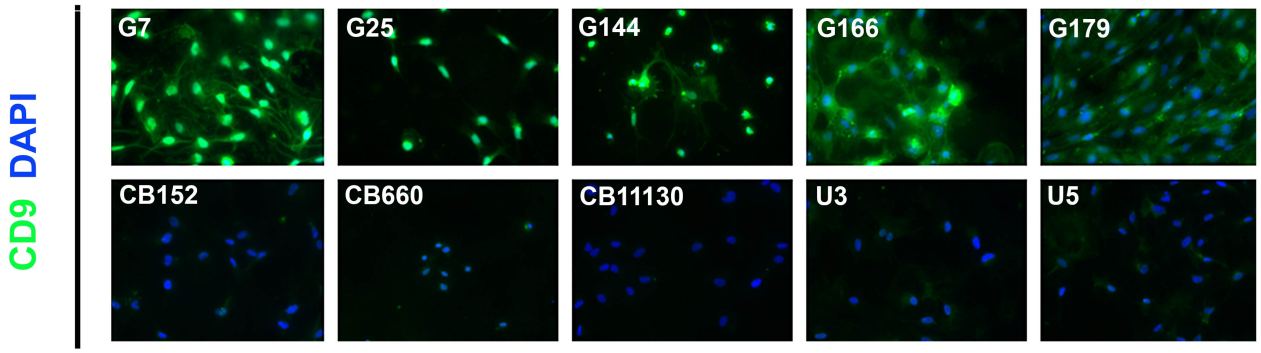
Figure 4

A

Data source	mRNA vs. Protein (Fig 3B)	Ligand-receptor interaction (Table 4)	jActive Mudule network (Fig 5A)	Gene regulatory network (Fig 5B)
Selected candidates	CD9,TNC, LGALS3, THY1	CD9, LGALS3	LGALS3, THY1	TNC, CD9







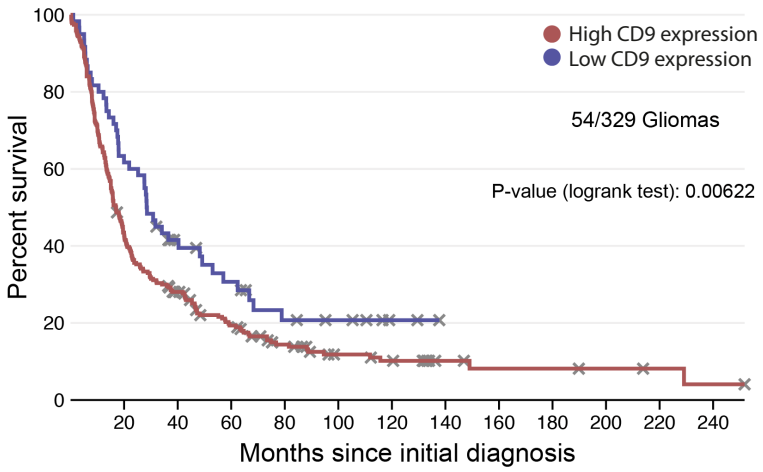


Figure 4. Validation of candidate markers in GNS cells.

(A) Inclusion criteria for proteins in experimental follow experiments. (**B**) Western blot analysis of CD9, LGALS3and TNC across 5 GNS and 5 NS cell lines showing consistent increased expression of CD9 in GNS cells. (**C**). Immunocytochemistry of CD9 (green) and nuclei (DAPI, blue) across 5 GNS and 5 NS cell lines. **(D)** Quantification of CD9 expression in GNS and NS cells derived from panel C.**(E)** Glioma patient survival analysis of CD9 using the Rembrandt database.

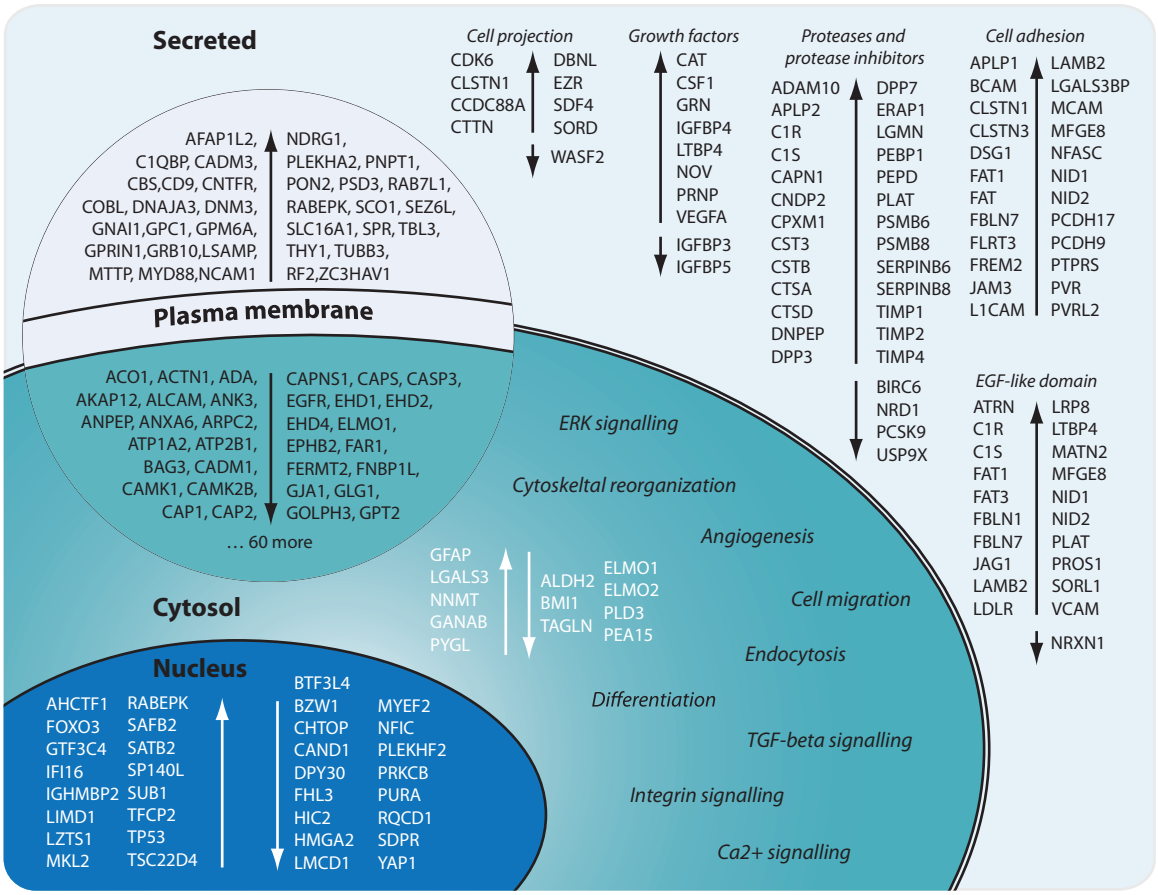


Figure 5.Model summarizing differentially expressed proteins and enriched processes.

The displayed proteins and processes summarize the combined results of GSEA projected on cellular localization. Arrows pointing upwards and downwards indicate up-regulated and down-regulated proteins in GNS cells compared to NS cells.

Table 1

Gene set feature	Annotated genes	DE genes	padj
A. Chromosome			
15	225	27	0.011
7	366	36	0.035
X	269	6	0.043
B. GO.BP			
cell differentiation	1001	97	0.000
endocytosis	176	29	0.000
neuron differentiation	444	53	0.000
integrin-mediated signaling pathway	31	9	0.004
NAD metabolic process	18	7	0.004
blood vessel development	187	24	0.011
regulation of cell migration	146	20	0.015
response to metal ion	108	16	0.019
cell-cell adhesion mediated by integrin	7	4	0.020
negative regulation of transforming growth factor beta receptor signaling pathway	17	5	0.040
positive regulation of calcium-mediated signaling	5	3	0.045
extracellular matrix organization	65	10	0.049
C. Panther pathway			
Integrin signalling pathway	113	26	0.000
Cytoskeletal regulation by Rho GTPase	45	9	0.023
D. Reactome pathway			
Cell-extracellular matrix interactions	11	10	0.000
Cell junction organization	34	13	0.000
L1CAM interactions	70	16	0.000
Regulation of cytoskeletal remodeling and cell spreading by IPP complex components	5	5	0.002
Response to elevated platelet cytosolic Ca2+	38	10	0.003
Localization of the PINCH-ILK-PARVIN complex to focal adhesions	4	3	0.004
Platelet activation, signaling and aggregation	101	16	0.008
NCAM signaling for neurite out-growth	35	9	0.009
Integrin cell surface interactions	45	10	0.009
c-src mediated regulation of Cx43 function and closure of gap junctions	3	3	0.023
ERK1 activation	3	3	0.023
Microtubule-dependent trafficking of connexons from Golgi to the plasma membrane	8	4	0.028
E. Wikipathway			
Focal Adhesion	117	18	0.006
Integrin-mediated cell adhesion	70	12	0.017
Regulation of Actin Cytoskeleton	92	14	0.020
Signaling of Hepatocyte Growth Factor Receptor	27	7	0.020
F. KEGG			
Focal adhesion	122	24	0.000
Regulation of actin cytoskeleton	123	24	0.000
Gap junction	45	12	0.000

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